All cells are equipped to sense changes in their environment and make adaptive responses according to the stimuli. Signal recognition usually occurs at the cell membrane (with the exception of steroid signalling) where the ligand, which can be a small molecule, a peptide or a protein, binds its cognate receptor. This results in a change in the conformation of the receptor which in turn can regulate the production of second messengers. Second messengers can now modulate specific pathways which control gene expression and modify various aspects of cell behaviour. The signalling cascade is terminated by the removal of second messenger and/or by desensitisation of the receptor to the extracellular signal.

Cyclic guanosine monophosphate (cGMP) was first identified in the rat urine and since then has emerged as an important second messenger regulating diverse cell processes. Subsequent to its discovery in mammalian cells, enzymes responsible for its synthesis (guanylyl cyclases), hydrolysis (phosphodiesterases) and its most common effectors (cGMP-dependent protein kinases) were identified. Guanylyl cyclases exist in two forms, cytosolic and membrane bound. Both have a conserved guanylyl cyclase domain, but differ in their choice of ligands, overall structure and tissue localization. It is now known that cytosolic and the membrane-bound forms are involved in eliciting distinct cellular responses.

Receptor guanylyl cyclase C (GC-C) was identified as the target for a family of heat-stable enterotoxin toxins (ST) produced by enterotoxigenic *E.coli*. Stable toxin-mediated diarrhoeas are observed frequently in infants and contribute significantly to the incidence of Travellers' Diarrhea. Early studies demonstrated that the effects of ST were mediated by an increase in intracellular cGMP levels in intestinal cells, and the receptor for ST was almost exclusively expressed in the apical microvilli of the intestinal brush-border epithelia. Effectors of cGMP in intestinal cells include protein kinase G (PKG), cyclic nucleotide gated ion channel 3 (CNG), and the cystic fibrosis transmembrane conductance regulator (CFTR). ST is an exogenous ligand which serves as a hyperagonist for GC-C, in comparison with the endogenous ligands guanylin and uroguanylin, which maintain fluid-ion homeostasis in the intestinal epithelia. The GC-C/cGMP signal transduction pathway also modulates intestinal cell proliferation along the crypt-villus axis by exerting a cytostatic effect on the epithelial cells, thereby regulating their turnover and neoplastic transformation.

The current study describes in molecular detail two signalling pathways, one impinging on and one emerging from GC-C, which regulate colonic cell proliferation. The first part identifies the cross-talk and cross-regulation of GC-C and c-src. The second part delves into the molecular basis of GC-C/cGMP-mediated cytostasis and its effect on colonic tumorigenesis.

Cross-talk between signalling pathways is believed to play a key role in regulating cell physiology. Phosphorylation of signalling molecules by protein kinases is frequently used as a means of achieving this cross-regulation. Aberrant hyperactivation of the c-src tyrosine kinase is an early event in the progression of colorectal cancer, and activated c-src specifically phosphorylates a number of proteins in the cell. It was found that c-src can phosphorylate GC-C in T84 colorectal carcinoma cells, as well as in the rat intestinal epithelia. Tyrosine phosphorylation of GC-C resulted in attenuation of ligand-mediated cGMP production; an effect which was reversed by chemical or transcriptional knockdown of c-src. These effects were found to be cell line-independent and relied only on the extent of c-src expression and activation in the cell.

Mutational analysis revealed GC-C to be phosphorylated on a conserved tyrosine residue (Y820) in the guanylyl cyclase domain. The sequence of GC-C around Y820 allowed for efficient phosphorylation by c-src, and indeed, kinase assays indicated that the affinity of c-src for the GC-C Y820 peptide was one of the highest reported till date. A phospho-mimetic mutation at this site, which mimics a constitutively phosphorylated receptor, resulted in a sharp reduction of guanylyl cyclase activity of the receptor, reiterating the inhibitory role of Y820 phosphorylation on GC-C activity. Phosphorylation of GC-C at Y820 generated a docking site for the SH2 domain of c-src which could interact and thereby co-localize with GC-C on the cell membrane. Intriguingly, this interaction resulted in activation of c-src, setting-up a feed-forward loop of inhibitory GC-C phosphorylation and c-src activation.

Treatment of colorectal carcinoma cells with ligands for GC-C reduces cell proliferation and inhibits tumorigenesis. It was observed that this cytostatic effect can be modulated by the status of c-src activation, and consequently, the fraction of tyrosine phosphorylated GC-C in these cells. Since activation of c-src is a frequent event in intestinal

neoplasia, phosphorylation of GC-C by active c-src may be one of the means by which the cytostatic effects of GC-C agonists (guanylin and uroguanylin) in the intestine are bypassed, thereby leading to cancer progression.

Colonisation of the gut with enteropathogenic microorganisms induces secretion of IFNγ from the host mucosal immune system, which subsequently activates c-src in intestinal epithelial cells. Ligand-stimulated activity of GC-C was found to be reduced in IFNγ treated cells. This could be one of the host defence mechanisms initiated in response to enterotoxigenic *E. coli* infection. These results provide the first evidence of cross-talk between a receptor guanylyl cyclase and a tyrosine kinase that results in heterologous desensitisation of the receptor.

Populations with a higher incidence of enterotoxigenic *E.coli* infections appear to be protected from intestinal neoplasia. It was found that mice lacking GC-C, and therefore unable to respond to ST, displayed an increased cell proliferation in colonic crypts and enhanced carcinogen-induced aberrant crypt foci formation, which is a surrogate marker for colorectal carcinogenesis. However, pharmacological elevation of cGMP was able to efficiently induce cytostasis even in GC-C knockout mice, indicating a key role for cGMP in regulating colonic cell proliferation. Through microarray analyses, genes regulated by ST-induced GC-C activation in T84 colorectal carcinoma cells were identified. Genes involved in a number of cellular pathways were differentially expressed, including those involved in signal transduction, protein and solute secretion, transcriptional regulation and extracellular matrix formation. One of the genes found to be significantly up-regulated was the cell-cycle inhibitor, p21. The increase in p21 expression was validated at both the transcript and protein level. This p53-independent up-regulation of p21 was coupled to the activation of the cGMP-responsive kinase, PKGII, since knockdown of PKGII using specific siRNAs abolished ST-induced p21 induction. Activation of PKGII led to phosphorylation and activation of the stress responsive p38 MAPK. Similar to what was seen following knockdown of PKGII, inhibition of p38 MAPK activity attenuated the up-regulation of p21 in response to cGMP, indicating that PKGII and p38 MAPK could be a part of a pathway regulating p21 expression. It was found that active p38 MAPK phosphorylated the

ubiquitous transcription factor SP1, enhancing its occupancy at the proximal p21 promoter. Therefore, SP1 could be one of the factors linking cGMP to transcription of the p21 mRNA.

Chronic activation of GC-C led to nuclear accumulation of p21 in colonic cells, which entered a quiescent state. These cells arrested in the G1 phase of the cell cycle, consequent to p21-dependent inhibition of the G1 cyclin-CDK complexes. A fraction of these quiescent cells stochastically initiated a cGMP-dependent senescence programme and displayed all the hallmarks of senescent cells, including flattened cell morphology, expression of SA-β galactosidase and formation of senescence-associated heterochromatic foci. Activation of senescence and loss of tumorigenicity in these cells was crucially dependent on the up-regulation of p21. This irreversible exit from the cell cycle due to cGMP-mediated activation of the PKGII/p38/p21 axis was well correlated with reduced colonic polyp formation in mice exposed to ST.

In summary, these observations may provide a possible explanation for the low incidence of colorectal carcinoma seen in countries with a high incidence of ST-mediated diarrhoea. Interestingly, c-src mediated tyrosine phosphorylation of GC-C prevented p21 accumulation following ligand application. The findings described in this thesis may have important implications in understanding the molecular mechanisms involved in the progression and treatment of colorectal cancer.